J Periodont Res 2006; 41; 193–199

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2005.00859.x

The 17-kDa sheath protein in enamel proteins induces cementum regeneration in experimental cavities created in a buccal dehiscence model of dogs

M. Kanazashi[1], K. Gomi[1], T. Nagano[1], T. Tanabe[2], T. Arai[1], M. Fukae[2]

Departments of [1]Periodontics and Endodontics, and [2]Biochemistry, School of Dental Medicine, Tsurumi University, Yokohama, Japan

Kanazashi M, Gomi K, Nagano T, Tanabe T, Arai T, Fukae M. The 17-kDa sheath protein in enamel proteins induces cementum regeneration in experimental cavities created in a buccal dehiscence model of dogs. J Periodont Res 2006; 41: 193–199.

Background and Objective: Commercially available enamel proteins, such as Emdogain®, are clinically used for periodontal regeneration. However, the real mechanisms behind the bioactivities of enamel proteins is still unclear, as enamel proteins have multicomponents. The purpose of this *in vivo* study was to identify the cementum regeneration-promoting factor in enamel proteins that is clinically used for periodontal regeneration to induce cementum-promotive and osteopromotive activities.

Material and Methods: Cementum regeneration, which is an important part of periodontal regeneration, was examined in experimental cavities prepared on a buccal dehiscence model of dogs. The purification of enamel protein with cementum regeneration activity was carried out by gel filtration and ion exchange chromatographies of newly formed secretory enamel.

Results: Cementum regeneration activity was found in the aggregate comprising 13–17-kDa sheath proteins along with a small amount of amelogenins, found in the newly formed secretory enamel. In these proteins, cementum regeneration activity was detected upon application of the 17-kDa sheath protein, but not by other lower molecular-weight sheath proteins and amelogenins. However, the purified 17-kDa sheath protein induced cementum regeneration activity only in a small area, although the regenerated cementum was thick. The activity of the 17-kDa sheath protein was believed not to have been a result of contamination by growth factors such as transforming growth factor-β1 (TGF-β1) found in the enamel protein, as the application of TGF-β1 induced weak cementum regeneration activity.

Conclusion: It is concluded that the 17-kDa sheath protein itself exhibits cementum regeneration activity, although other factors may be needed to demonstrate its full ability.

Mikimoto Kanazashi, Department of Periodontics and Endodontics, School of Dental Medicine, Tsurumi University, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama 230-8501, Japan
Tel: +81 45 581 1001
Fax: +81 45 583 8401
email: gomi-k@tsurumi-u.ac.jp

Key words: buccal dehiscence model; cementum regeneration; enamel proteins; sheath protein

Accepted for publication October 4, 2005

It is believed that enamel proteins have bioactivities, such as the induction of osteogenesis and cementogenesis, in addition to their involvement in enamel formation. Periodontium has been reported to be regenerated in experimental cavities created on a buccal dehiscence model in monkeys, after the application of porcine enamel matrix proteins (1–3). It has also been demonstrated that enamel matrix derivatives stimulate the proliferation and differentiation of human periodontal ligament cells (4) and enhance bone formation (5). Based on these results, commercially available enamel proteins, such as Emdogain®, are used clinically for periodontal regeneration. However, the real mechanisms behind the bioactivities of enamel proteins are still unclear, as enamel proteins have multicomponents that comprise amelogenin as the major structural protein besides enamelin (6), and sheathlin (7) [a homologue of ameloblastin (8) or amelin (9)]. These are degraded during the mineralization process by two proteinases – enamelysin (10) and KLK4 (which is also known as EMSP-1) (11). Therefore, the immature enamel matrices contain many enamel protein derivatives.

Recently, bone morphogenetic protein (BMP)-like activity has been detected in porcine enamel extracts using ST2 cells, a mouse bone mallow stromal cell line (12), and transforming growth factor- β (TGF-β)-like activity has also been detected in studies using oral epithelial and fibroblastic cells (13). The existence of these growth factors was determined by a specific luciferase reporter gene assay in porcine enamel protein (14). The BMP-like activity increased the alkaline phosphatase (ALP) activity of ST2 cells and this induced ALP activity was inhibited by the action of noggin. The TGF-β-like activity in enamel matrix protein increases the ALP activity of human periodontal ligament (HPDL) cells, promotes cell differentiation and, finally, induces mineralization (15). The relationship between these growth factor-like activities found in enamel proteins, and bioactivities such as the induction of osteogenesis and cementogenesis in periodontal regeneration, needs to be elucidated.

In the present study, we report that cementum regeneration, which is an important part of periodontal regeneration, occurred in experimental cavities created in the buccal dehiscence model in the dog *in vivo* system, upon application of the fraction containing sheath proteins.

Material and methods

All experimental procedures involving the use of animals were reviewed and approved by the Institutional Animal Care Program of Tsurumi University.

Recombinant TGF-β1 was purchased from R & D Systems, Inc. (Minneapolis, MN, USA).

Extraction of the enamel proteins

Unless stated otherwise, all procedures were carried out at 4°C or in ice-cold conditions. Permanent incisor tooth germs were dissected from the fresh mandibles of 6-month-old pigs. From secretory enamel, a thick layer of ≈30 μm was scraped from the outermost layer beneath the ameloblast cell layer to serve as the outer-layer sample (newly formed enamel). The inner-layer sample was also prepared from the secretory enamel, after removal of a superficial layer of ≈60 μm thickness (16).

The protein was extracted from each pooled sample after homogenizing for 40 s at ≈6000 r.p.m. using a Polytron homogenizer. The neutral-soluble fraction and the alkaline-soluble fraction were sequentially extracted by homogenizing in 0.05 M Sörensen buffer (pH 7.4) and in 0.05 M carbonate-bicarbonate buffer (pH 10.8), respectively (17), and extraction using the same buffer was repeated three times. Over 95% of total protein was extracted by these sequential extractions from the immature enamel (18,19).

Purification of sheath proteins

The alkaline-soluble fraction of a newly formed enamel sample was gel filtrated into four fractions (fr.1–4) using a column (2.6 × 100 cm) of Sephadex G-100 (Pharmacia Biotech, Uppsala, Sweden), which was equilibrated with 0.05 M carbonate-bicarbonate buffer (pH 10.8). As the first eluted peak (fr.1) contained 89-kDa enamelin and the aggregate comprised 13–17-kDa sheath proteins along with a small amount of 20–25-kDa amelogenins, it was separated on a DEAE ion exchange HPLC column (9 × 100 mm) of EXPRESS-ION™ EXCHANGER Q (Whatman; Whatman International Ltd, Springfield Mill, UK) equilibrated with 0.05 M Tris-HCl containing 6 M urea (pH 7.4). The column was eluted with a linear NaCl gradient (0–1.2 M). The fraction containing sheath proteins was further separated by a gel filtration recycle system using a column (2.5 × 95 cm), Cellulofine GCL-2000 (Chisso Ltd, Makuhari, Japan), or double-tundem columns (7.5 mm I.D. × 60 cm), TSKgel G3000PW (TOSOH, Tokyo, Japan) equilibrated in 0.05 M Tris-HCl containing 4 M guanidine-HCl (pH 7.4).

Animals and surgical procedure

Eight-week histological analysis was performed on buccal dehiscence-type bone defects in canine mandibles (20).

A total of seven 2-year-old male beagle dogs (weighing ≈15 kg) were used to detect the cementum regeneration activities after application of several enamel protein samples. They had an intact dentition and healthy periodontium. Five dogs were used for fr.1 (the first fraction eluted by Sephadex G-100 column), with Emdogain® (EMD; BIORA AB, Malmö, Sweden) as a positive control and distilled water as a negative control. Two dogs were used to study the effect of partially purified 17-kDa and 15-kDa sheath protein, and recombinant TGF-β1 .

The surgical preparation of buccal dehiscence-type bone defects was carried out according to the modified method of Hammarström (1). The procedures were conducted under general anesthesia, induced and maintained by an endotracheal tube with 1.5–2.0% halothane (Takeda Yakuhin Co. Ltd, Osaka, Japan) in 100% oxygen, after premedication with an intramuscular injection of a mixture of ketamine HCl (9.8 mg/kg; Sankyo Co. Ltd, Tokyo, Japan) and Xylazine HCl

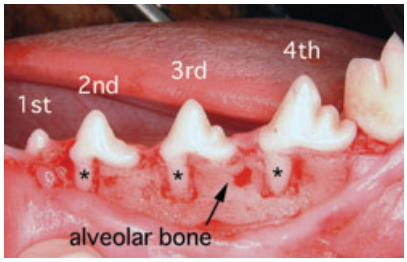

Fig. 1. Buccal dehiscence-type bone defects created along the premolar roots of beagle dogs. The asterix marks show the exposed dentin surface after complete removal of the buccal alveolar bone, periodontal ligament and cementum, by means of a dental bur.

(0.7 mg/kg; Bayer Co. Ltd, Tokyo, Japan).

Contralateral buccal mucoperiosteal flaps were raised after making an intracrevicular incision, extending from the first mandibular premolar to the first mandibular molar, as shown in Fig. 1. After removal of the buccal alveolar bone on the mesial root of premolars, except the first premolar, the periodontal ligament and cementum of these areas were completely removed by means of a dental bur. At the apical end of the defect, a notch was created to identify the apical extension of the defect during histological analysis. The distance from the cervical margin to the apical end of the defect was standardized to ≈ 5 mm. The exposed dentin surfaces were conditioned for 2 min, by means of cotton pellets soaked in a 19% EDTA solution, and then carefully rinsed with sterile saline. Then, 50 μg of each enamel protein sample and EMD was dissolved in 100 μl of cold sterile distilled water and applied over the entire buccal surface area of the exposed roots. In experiments with recombinant TGF-β1, a concentration of 2.0 μg/100 μl was used. Finally, the mucoperiosteal flaps were repositioned to their original level and sutured.

After 8 weeks, the dogs were killed after anesthetization with sodium pentobarbital and their heads were fixed by perfusion with 0.1 M sodium cacodylate buffer (pH 7.4) containing 4% paraformaldehyde and 1% glutaradehyde. Tissue blocks, which included teeth, bone, and tissue, were dissected and then rapidly demineralized in 10% formic acid containing 0.06 M sodium citrate and 0.1 M citric acid. All specimens were dehydrated in a graded series of alcohols and embedded in paraffin. Serial sections (5 μm thick) were made and stained with hematoxylin/eosin for examination by light microscopy.

Histological analysis

The length and thickness of cementum regenerated after application of fractionated and purified enamel protein samples and EMD were measured, using a computer, from the histological results. The length of the regenerated cementum was the straight distance between the apical end of the defect and coronal extension of the new cementum, as well as to the crest of the new alveolar bone. The thickness of cementum was also measured at the coronal end, at the apical end and between them. The data obtained were analyzed statistically by the *t*-test.

Analytical methods

Acrylamide gel electrophoreses were carried out using 15% polyacrylamide slab gels containing 1% sodium dodecyl sulfate (SDS), according to the method of Laemmli (21), and stained with Coomassie Brilliant Blue R-250.

Amino acid sequence analyses were carried out using the Shimadzu protein sequencer, PPSQ-23 A (Shimadzu Co., Kyoto, Japan).

Results

Cementum regeneration activity of each fraction

Cementum regeneration activity was found in the outer-layer (newly formed) enamel sample rather than in the inner-layer enamel sample on the 8-week histological samples of buccal dehiscence-type bone defects created along the roots of canine mandible. In the newly formed enamel sample, the cementum regeneration activity was found in the alkaline-soluble fraction, but not in the neutral-soluble fraction.

The alkaline-soluble fraction of newly formed enamel was separated into four fractions by Sephadex G-100 gel filtration (Fig. 2). The cementum regeneration activity in these fractions was examined and observed in the first eluted peak (fr.1), but not in the other fractions, which contained only amelogenins and their derivatives.

Figure 3 shows the histological analysis of cementum regeneration 8 weeks following the application of fr.1 and EMD. Both samples regenerated cementum from the notch to the cervical margin. The distance from the apical end of the bone defect to the new cementum was 6.24 ± 0.40 mm, 6.15 ± 0.43 mm and 4.17 ± 0.79 mm for fr.1, EMD and the control, respectively. There were significant differences between the control and fr.1 or EMD, but no significant difference between fr.1 and EMD. The bone height from the apical end of the bone defect was 1.69 ± 0.45 mm, 1.41 ± 0.54 mm and 1.09 ± 0.46 mm at fr.1,

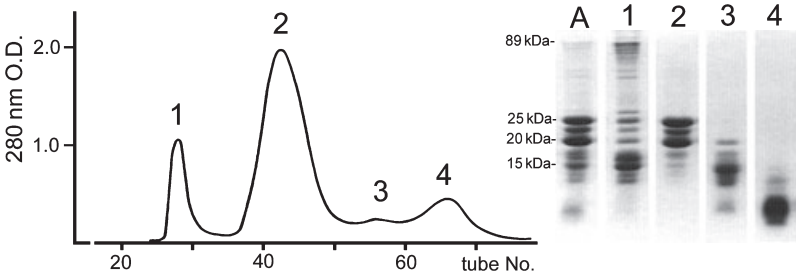

Fig. 2. Protein profile of the alkaline-soluble fraction (fr.) of newly formed enamel obtained by Sephadex G-100 gel filtration. (A) Alkaline-soluble fraction of newly formed enamel: 1, fr.1; 2, fr.2; 3, fr.3; 4, fr.4. Fr.1 contained sheath proteins with a molecular weight lower than that of 20–25-kDa amelogenins, as shown in fr.2. Fr.3 and fr.4 contained amelogenin derivatives.

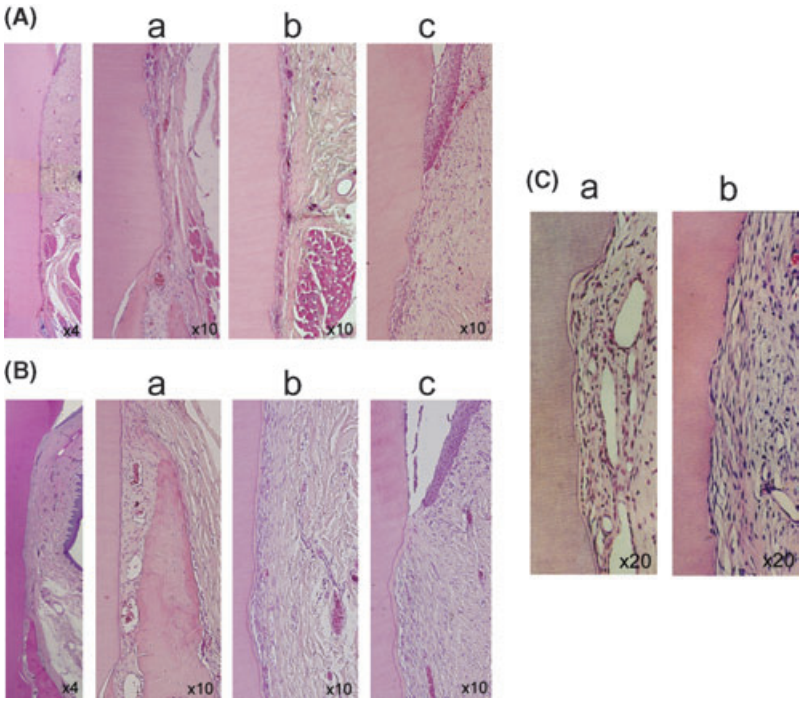

Fig. 3. Light micrographs showing cementum regeneration 8 weeks following the application of (A) Emdogain® (EMD), (B) fraction 1 (fr.1) and (C) fraction 2 (fr.2) on the buccal dehiscence-type bone defects created along the premolar roots of canine mandibles. (A) Whole area after the application of EMD. (B) Whole area after the application of fr.1. (a) Bottom around the notch created to enable identification of the apical extension of the defect; (b) middle; and (c) upper part around the cervical margin. (C) comparison between EMD and fr.2 (a) Middle area of EMD application; and (b) middle area of amelogenins (fr.2) application.

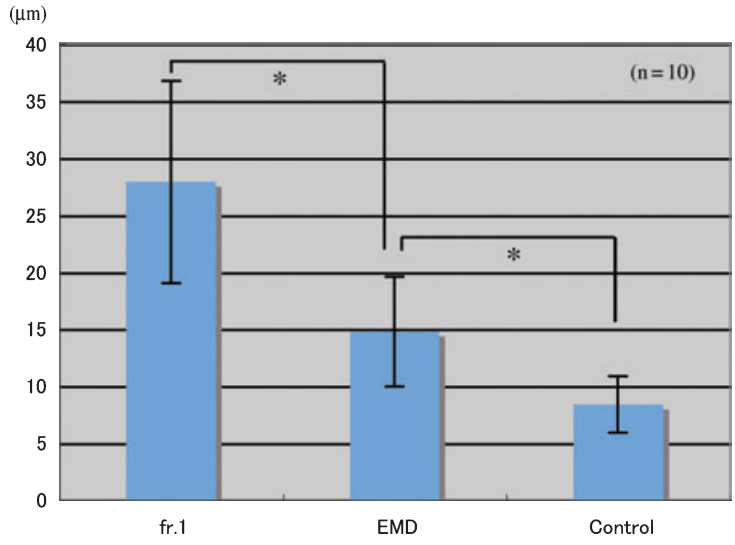

Fig. 4. The thickness of regenerated cementum by fraction 1 (fr.1), Emdogain® (EMD) and the control to evaluate the activity of cement genesis. Statistically significant differences were found between each group (*$p < 0.001$). $n = 10$.

EMD and the control, respectively. A significant difference was found only between fr.1 and the control.

The thickness of cementum was measured at three points (apical, middle, coronal) of each sample. The average thickness of cementum was 27.88 ± 8.85 μm, 14.77 ± 4.81 μm and 8.37 ± 2.48 μm for fr.1, EMD and the control, respectively. Statistically significant differences were recognized between each group (Fig. 4). Fr.1 induced formation of thick acellular cementum that was well attached to the dentin. Numerous collagen fiber bundles, arranged as in normal periodontium, were produced from the regenerated cementum (Fig. 3B). The cementum regeneration activity (thickness of cementum) of EMD, which was the positive control, was obviously weaker than in fr.1 (Figs 3A and 4). The application of water used as the negative control showed negligible cementum regeneration activity.

The fractions, except fr.1, separated by Sephadex G-100 gel filtration containing amelogenins or their derivatives (fr.2) did not show cementum regeneration activity (Fig. 3C, b). In this case, the thickness and length of regenerated cementum were not measured because few experimental cavities were used for amelogenin.

It was shown, by SDS electrophoresis, that fr.1 contained 70–89-kDa enamelin and 13–17-kDa sheath proteins, along with a small amount of 20–25-kDa amelogenins. These sheath proteins formed the aggregate (Fig. 1). The lower-molecular-weight sheath proteins were eluted at an elution position of high-molecular-weight protein. From these findings, it was confirmed that the aggregate contained 13–17-kDa sheath proteins, along with a small amount of amelogenins eluted prior to 89-kDa enamelins on the gel filtration system of double tundem TSKgel G3000PW columns under alkaline pH conditions (0.05 M carbonate-bicarbonate buffer, pH 10.8) (data not shown).

The aggregate fraction was separated from fr.1 by ion exchange chromatography (Fig. 5A) and examined for its cementum regeneration activity. The fraction induced the formation of

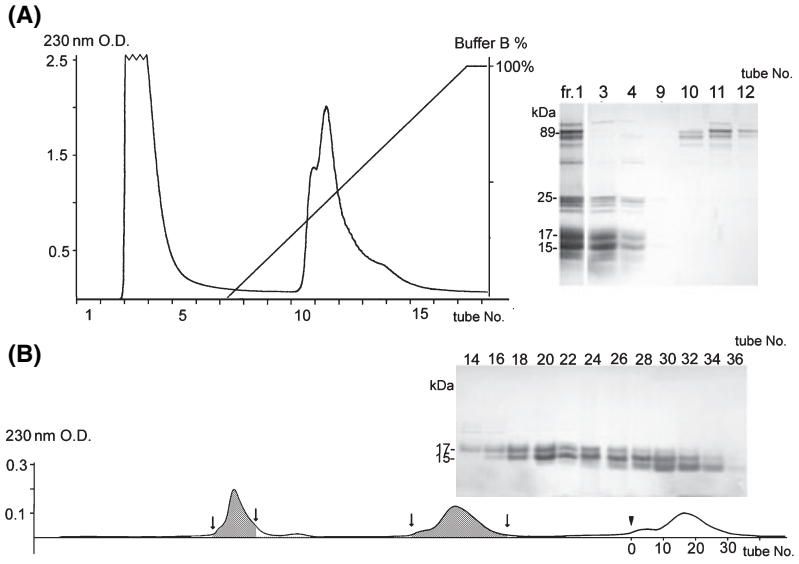

Fig. 5. (A) Protein profile of fraction 1 (fr.1) further fractionated by DEAE ion-exchange HPLC using an EXPRESS-ION^{TM} EXCHANGER Q column in 6 M urea solution (pH 7.4). The sheath proteins were eluted, without retard, along with a small amount of amelogenins. The flow rate was 1.0 ml/min, and 2.0 ml was collected in each tube. (B) Protein profile of the sheath protein fraction, further separated by the gel filtration recycling system using a Cellulofine GCL-2000 column in 4 M guanidine solution (pH 7.4). Arrows show the connection position for recycling. The arrowhead shows the position connecting to the fraction collector. The flow rate was ≈20 ml/h, and 4.0 ml was collected for one tube. The sodium dodecyl sulphate (SDS) electrophoresis pattern shows the separation between the 17-kDa sheath protein and the 15-kDa sheath protein. Recycling was carried out 12 times, using double-tundem columns of TSKgel G3000PW, in order to obtain homogenous sheath protein.

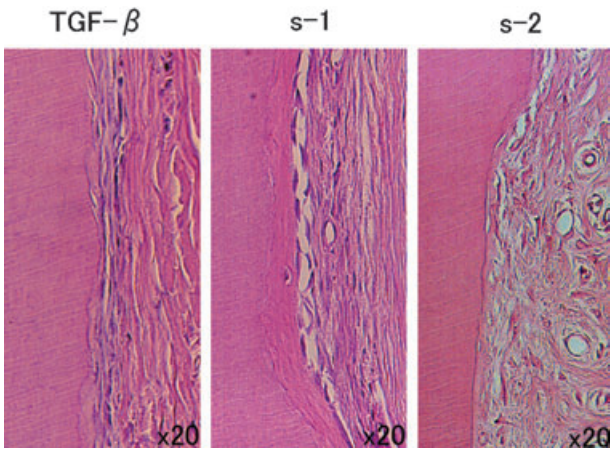

Fig. 6. Light micrographs showing the cementum regeneration 8 weeks following the application of transforming growth factor-β1 (TGF-β), the 17-kDa sheath protein (s-1) and the 15-kDa sheath protein (s-2), on the buccal dehiscence-type bone defects created along the premolar roots of canine mandibles. The 17-kDa and 15-kDa sheath protein solutions were applied at a concentration of 50 μg/100 μl, and recombinant TGF-β1 was applied at a concentration of 2.0 μg/100 μl. These were dissolved in distilled water.

thick acellular cementum on the experimental cavities, although the cementum regeneration occurred in a smaller area than was observed with the fr.1 application (data not shown). These results indicated that there was cementum regeneration activity in the sheath protein fraction, but not in the enamelins and the amelogenins. Therefore, the sheath proteins were purified by a gel filtration recycle system using a column of Cellulofine GCL-2000 (Fig. 5B) or the TSKgel G3000PW equilibrated in 0.05 M Tris-HCl buffer containing 4 M guanidine-HCl (pH 7.4).

Cementum regeneration activity of 17-kDa and 15-kDa sheath proteins

Partially purified 17-kDa and 15-kDa sheath proteins were tested for their cementum regeneration activity using the experimental cavities created on canine mandible (Fig. 6). The 15-kDa sheath protein showed minimal cementum regeneration activity. TGF-β1 also induced cementum regeneration, but the amount of this was small. This indicated that the cementum regeneration activity of the 17-kDa sheath protein was not a result of contamination with the TGF-β1 found in the enamel matrix proteins. The thickness of cementum was 10.41 ± 5.92 μm, 31.77 ± 3.78 μm and 8.13 ± 2.06 μm with TGF-β, 17 kDa and 15 kDa, respectively. Statistical analysis was not performed for these data because only four experimental cavities were used for each protein sample. The scarcely regenerated cementum induced by application of the 15-kDa sheath protein may be a result of the contamination of the 15-kDa sheath protein sample by the 17-kDa sheath protein.

When the further purified 17-kDa sheath protein was histologically analysed for cementum regeneration after 8 weeks, it had induced thick acellular cementum near the apical end of the defect, but the cementum regeneration was scarce near the coronal end. Epithelial downgrowth was sometimes observed. These were different from the results observed following the application of fr.1.

The amino acid sequence analyses of purified sheath proteins demonstrated that the 17- and 15-kDa sheath proteins corresponded to the N-terminal side of sheathlin (7) containing 170 and 130 amino acids, respectively (Fig. 7). These indicated that the cementum

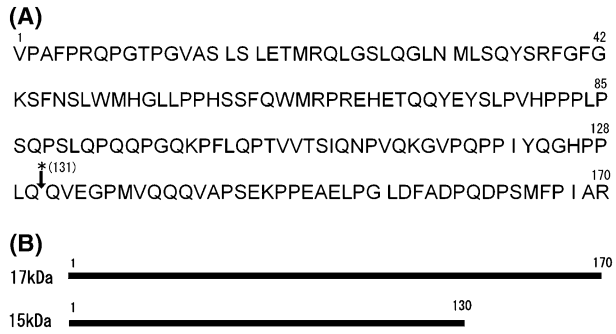

Fig. 7. Amino acid sequence of the 17-kDa sheath protein (A) and the relationship of the 17-kDa and 15-kDa sheath protein (B). The 17-kDa sheath protein sequence is based upon partial amino acid sequences of the purified sheath proteins and the deduced sequence of parent protein, sheathlin, as previously described (7). The asterisk denotes the C-terminal end of the 15-kDa sheath protein. The arrow indicates a cleavage site.

regeneration activity found in the 17-kDa sheath protein was located in its C-terminal side peptide, which was not found in the 15-kDa sheath protein.

Discussion

It is shown that the application of porcine enamel matrix in experimental cavities created in the roots of incisors of monkeys induces the formation of acellular cementum (1,2). The idea is produced based on the fact that coronal acellular extrinsic fiber cementum is formed on the enamel surface in a number of species. It indicates that the enamel matrix proteins have the potential to induce regeneration of the same type of cementum. It is also shown that the EMDs have bioactivities, such as the induction of osteogenesis and cementogenesis (4,5). Based on these results, the enamel matrix proteins are used for repairing the periodontal ligament of periodontitis, as one periodontal regeneration remedy (3,22). However, when the enamel proteins are applied for the treatment of periodontitis, periodontal ligament regeneration is not always accomplished as the expected result and leaves much room for improvement.

The problem remaining to be solved is elucidation of the real form of bioactivities contained in the enamel proteins. The researchers who developed the EMD expect that the bioactivities are caused by the amelogenin family, a major component in enamel matrix. However, the amelogenins and their derivatives separated by ammonium sulfate precipitation fractionation (20) or gel filtration (23) have no cementum regeneration activity, as determined by histological analysis using experimental defects created in a buccal dehiscence model of dogs.

In this article, the cementum regeneration-promoting factor in porcine enamel protein was investigated using buccal dehiscence bone defects surgically created in canine mandibles. Compared with the application of EMD, which showed obvious cementum regeneration activity, as reported previously (1–3), stronger cementum regeneration activity was found reproducibly in the fr.1 separated from the alkaline-soluble fraction of newly formed secretory enamel. The fr.1 consisted of enamelins and sheath proteins, which formed an aggregate together with a small amount of amelogenins. The amelogenins showed no cementum regeneration activity. This supports the previously reported findings that amelogenins, when separated by ammonium sulfate fractionation, show no cementum regeneration activity (20). The enamelins also showed no cementum regeneration activity. In fact, it was determined, from histological analysis, that the 17-kDa sheath protein exhibited cementum regeneration activity.

However, further purified 17-kDa sheath protein induced a smaller area of cementum regeneration on the dentin surface of experimental cavities created on premolar medial roots, and the regenerated cementum was thick. This may have occurred because water was employed for dissolution of the sample, not propylene glycol alginate, as used for EMD (2). Moreover, it is also possible that the smaller area of cementum regeneration was caused by the lack of amelogenins, because amelogenin may act as a carrier of the cementum regeneration-promoting factor.

The sheath protein was first identified as 13–17-kDa nonamelogenin protein (16) and named based on the evidence that it accumulates in the enamel sheath of immature enamel matrices of pig (24,25) and calf (26). The parent protein of the sheath protein is cloned as the sheathlin (7), which is a homologue of ameloblastin (8) or amelin (9). It has been shown that the sheathlin secreted from ameloblasts is degraded immediately into three pieces, namely sheath proteins, stains all positive protein (M. Fukae, unpublished) and Ca-binding protein (27,28). As the sheath protein is further degraded and its molecular weight was lower at the inner layer of secretory enamel, it can be assumed that the 17-kDa sheath protein is found only in the newly formed enamel. This confirms that the cementum regeneration activity was found only in the newly formed enamel.

Recently, BMP-like and TGF-β-like activities were observed in porcine enamel extracts, using ST2 and HPDL cells in a cell culture system *in vitro* (12–15). The TGF-β-like activity in the enamel matrix protein increases the ALP activity of HPDL cells, promotes their cell differentiation and induces the occurrence of mineralization during 15 d of culture, while BMP-2 decreases the ALP activity of HPDL cells (15). This indicates that these growth factors may affect the regeneration of cementum on the experimental cavities created on the buccal dehiscence model of dogs. However, TGF-β1 induced a weak cementum regeneration activity on histological analysis, providing evidence that the cementum regeneration activity found in the 17-kDa sheath protein is not caused by contamination with TGF-β1.

It has been shown in the cell culture system that the 17-kDa sheath protein increases the ALP activity of HPDL cells and the indication of cell differentiation, but the 15-kDa sheath protein does not (23). As previous studies

have demonstrated the important role played by ALP in acellular cementum formation, the findings of the present study suggest that this fraction is responsible for the induction of regeneration of cementum (29). The peptide synthesized based on the amino acid sequence of the C-terminal side peptide of 17-kDa sheath protein, which is not found in the 15-kDa sheath protein, appears to increase the ALP activity of the HPDL cells. It is concluded that the 17-kDa sheath protein itself has the physiological function of cementum regeneration activity along with cytodifferentiation, although other factors, such as amelogenin as a carrier of the cementum regeneration-promoting factor, may be needed to demonstrate its real ability.

References

1. Hammarström L. Enamel matrix, cementum development and regeneration. *J Clin Periodontol* 1997;**24:**658–668.
2. Hammarström L, Heiji L, Gestrelius S. Periodontal regeneration in a buccal dehiscence model in monkeys after application of matrix proteins. *J Clin Periodontol* 1997;**24:**669–677.
3. Heiji L. Periodontal regeneration with matrix derivative in one human experimental defect. A case report. *J Clin Periodontol* 1997;**24:**693–696.
4. Gestrelius S, Andersson C, Lindström D, Hammarström L, Somerman MJ. *In vitro* studies on periodontal ligament cells and enamel matrix derivative. *J Clin Periodontol* 1997;**24:**685–692.
5. Boyan BD, Weesner TC, Lohmann CH *et al*. Porcine fetal enamel matrix derivative enhances bone formatic induced by demineralized freeze dried bone allograft *in vivo*. *J Periodontol* 2000;**71:**1278–1286.
6. Hu C-C, Fukae M, Uchida T *et al*. Cloning and characterization of porcine enamelin mRNAs. *J Dent Res* 1997;**76:**1720–1729.
7. Hu C-C, Fukae M, Uchida T *et al*. Sheathlin: cloning, cDNA/polypeptide sequences, and immunolocalization of porcine enamel sheath proteins. *J Dent Res* 1997;**76:**648–657.
8. Krebsbach PH, Lee SK, Matsuki Y, Kozak CA, Yamada KM, Yamada Y. Full-length sequence, localization, and chromosomal mapping of ameloblastin: a novel tooth-specific gene. *J Biol Chem* 1996;**271:**4431–4435.
9. Cerny R, Slaby I, Hammarström L, Wurtz T. A novel gene expressed in rat ameloblasts codes for proteins with cell binding domains. *J Bone Miner Res* 1996;**11:**883–891.
10. Fukae M, Tanabe T, Uchida T *et al*. Enamelysin (matrix metalloproteinase-20): localization in the developing tooth and effects of pH and calcium on amelogenin hydrolysis. *J Dent Res* 1998;**77:**1580–1588.
11. Simmer JP, Fukae M, Tanabe T *et al*. Purification, characterization, and cloning of enamel matrix serine proteinase 1. *J Dent Res* 1998;**77:**377–386.
12. Iwata T, Morotome M, Tanabe T, Fukae M, Ishikawa I, Oida S. Noggin blocks osteoinductive activity of porcine enamel extracts. *J Dent Res* 2002;**81:**387–391.
13. Kawase T, Okuda K, Momose M, Kato Y, Yoshie H, Burns DM. Enamel matrix derivative (EMDOGAIN®) rapidly stimulates phosphorylation of the MAP kinase family and nuclear accumulation of smad2 in both oral epithelial and fibroblastic human cells. *J Periodont Res* 2001;**36:**367–376.
14. Suzuki S, Nagano T, Yamakoshi Y *et al*. Enamel matrix derivative gel stimulates signal transduction of BMP and TGF-β. *J Dent Res* 2005;**84:**510–514.
15. Nagano T. Effects of fractionated porcine enamel proteins on osteogenesis in human periodontal ligament (HPDL) cells *in vitro* [in Japanese]. *J Jpn Soc Periodontol* 2003;**45:**384–393.
16. Fukae M, Tanabe T, Uchida T, Shimizu M. The localization and characterization of proteinases for the initial cleavage of porcine amelogenin. *Calcif Tissue Int* 1992;**51:**213–217.
17. Fukae M, Tanabe T. Nonamelogenin components of porcine enamel in the protein fraction free from the enamel crystals. *Calcif Tissue Int* 1987;**40:**286–293.
18. Fukae M, Tanabe T. Degradation of enamel matrix proteins in porcine secretory enamel. *Connect Tissue Res* 1998;**39:**123–129 [427–433].
19. Fukae M. Amelogenesis – three-dimensional structure of amelogenin micells and their degradation by a cascade system. *Proc Int Symp Oral Sci Tsurumi Univ Sch Den Med* 2001;**1:**73–78.
20. Kanazashi M, Gomi K, Tanabe T, Nagano T, Fukae M, Arai T. Periodontal regeneration activity of protein fractions obtained from the enamel matrix derivatives by the ammonium sulfate fractionation [in Japanese]. *Tsurumi Univ Dent J* 2004;**30:**225–230.
21. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;**227:**680–688.
22. Heijl L, Heden G, Svärdström G, Östgren A. Enamel matrix derivative (EMDOGAIN®) in the treatment of intrabony periodontal defects. *J Clin Periodontol* 1997;**24:**705–714.
23. Fukae M, Kanazashi M, Nagano T, Tanabe T, Oida S, Gomi K. Porcine sheath proteins show periodontal ligament regeneration activity. *Eur J Oral Sci* 2005 (in press).
24. Uchida T, Tanabe T, Fukae M, *et al*. Immunochemical and immunohistochemical studies, using antisera against 25kDa amelogenin, 89kDa enamelin and the 13–17kDa nonamelogenins, on immature enamel of the pig and rat. *Histochemistry* 1991;**96:**129–138.
25. Uchida T, Fukae M, Tanabe T, *et al*. Immunochemical and immunocytochemical study of a 15kDa non-amelogenin and related proteins in the porcine immature enamel: proposal of a new group of enamel proteins 'Sheath Proteins'. *Biomed Res* 1995;**16:**131–140.
26. Fukae M, Tanabe T, Uchida T, Yamakoshi Y, Shimizu M. Enamelins in the newly formed bovine enamel. *Calcif Tissue Int* 1993;**53:**257–261.
27. Fukae M, Tanabe T. ^{45}Ca-labeled proteins found in porcine developing dental enamel at an early stage of development. *Adv Dent Res* 1987;**1:**261–266.
28. Yamakoshi Y, Tanabe T, Oida S, Hu C-C, Simmer JP, Fukae M. Calcium binding of enamel proteins and their derivatives with emphasis on the calcium-binding domain of porcine sheatthlin. *Arch Oral Biol* 2001;**46:**1005–1014.
29. Beertsen W, VandenBos T, Everts V. Root development in mice lacking functional tissue non-specific alkaline phosphatase gene: inhibition of acellular cementum formation. *J Dent Res* 1999;**78:**1221–1229.